Escherichia coli O157:H7 gene expression in the presence of catecholamine norepinephrine

Scot E. Dowd

United States Department of Agriculture, Agriculture Research Service, Livestock Issues Research Unit, Lubbock, TX, USA

Correspondence: Scot E. Dowd, USDA-ARS-LIRU 1604 E, FM 1294 Lubbock, TX 79403, USA. Tel.: +806 746 5356, ext. 122; fax: +806 746 5028; e-mail: sdowd@lbk.ars.usda.gov

Received 6 April 2007; revised 4 May 2007; accepted 4 May 2007.
First published online 16 June 2007.

DOI:10.1111/j.1574-6968.2007.00800.x

Editor: Rob Delahay

Keywords
pathogenesis; neuroendocrine; virulence; norepinephrine; *Escherichia coli*.

Abstract

Various forms of host stresses (e.g. physiological, psychological) are thought to influence susceptibility to pathogenic microorganisms. Catecholamines such as norepinephrine are released into the GI environment during acute stress and may influence the infective process of bacterial pathogens associated with the GI tract. To examine the effects of norepinephrine on expression of virulence factors in *Escherichia coli* O157:H7, the clinical-type isolate EDL933 (ATCC 43895) was grown in serum-Standard American Petroleum Institute media in the presence or absence of norepinephrine. After 5 h of exposure to norepinephrine, treatment and control cultures (not exposed to norepinephrine) were harvested, their RNA isolated, and gene expression evaluated. There was a dramatic increase in the expression of virulence factor transcripts including *stx*1, *stx*2, and *eae*. Also induced were transcripts involved in iron metabolism. Conversely, there was comparative repression of iron acquisition and phage shock protein-related transcripts in the presence of norepinephrine. Novel observations from these data suggested that exposure to norepinephrine induced glutamate decarboxylase acid resistance as well as an SOS response in *E. coli* O157:H7. The results corroborate many of the previous findings detailed in the literature and provide new observations that could expand the scope of microbial endocrinology.

Introduction

It has been reported (Lyte & Ernst, 1992; Lyte & Nguyen, 1997; Chen *et al.*, 2003; Green *et al.*, 2004) that there are direct neuroendocrine system (adrenergic) innervations of the GI tract, which may influence the outcome of *Escherichia coli* O157:H7 infection when activated by acute stress. One of the primary biochemicals of interest in the relatively new area of microbiology, termed microbial endocrinology, has been the catecholamine norepinephrine (Jones & Shapiro, 1963; Green *et al.*, 2003; Lyte, 2004a, b). What is not widely recognized is that norepinephrine- and dopamine-containing sympathetic nerve terminals are also widely distributed throughout the GI tract, where they make up a vital component of the enteric nervous system (ENS) (Costa *et al.*, 2000). Norepinephrine has been shown to spill into the small intestine environment, via the ENS, during acute stress (Eisenhofer *et al.*, 1995; Aneman *et al.*, 1996). For the host, the effects of norepinephrine release help modulate homeostasis during actual or perceived stress (Monjan & Collector, 1977). Interestingly, up to half of the norepinephrine within the mammalian body may be synthesized and utilized within the ENS as noted in a previous review (Lyte, 2004a).

There is a growing body of research indicating that the host microbiota, including commensals and pathogens, may respond to the presence of catecholamines. Norepinephrine has been shown to be a beneficial growth adjuvant to *E. coli* in serum-based media (Kinney *et al.*, 2000) with a primary effect of enhancing iron acquisition (Bowdre *et al.*, 1976; Freestone *et al.*, 2000). Further tying together the virulence systems of *E. coli* O157, with an norepinephrine-influenced environment within the GI tract, it has been shown that norepinephrine enhances growth and iron acquisition, effects motility, increases toxin expression, and promotes attachment of *E. coli* O157 *in vivo* (Lyte *et al.*, 1996a, 1997; Freestone *et al.*, 1999, 2000; Chen *et al.*, 2003; Green *et al.*, 2004). Thus, a link between the noradrenergic stress response in the host and the pathogenesis of *E. coli* O157:H7 has been described.

There are mechanisms of genetic regulation related to norepinephrine and its influence on the pathogenesis of *E. coli* O157 that remain to be elucidated. As an initial attempt to corroborate previous findings and identify new

research directions, gene expression has been evaluated in an *E. coli* O157:H7 EDL933-type isolate in serum-Standard American Petroleum Institute (SAPI) media, either in the presence or in the absence of norepinephrine. By comparing expression profiles in treatment and control cultures, it was hoped that new putative norepinephrine regulatory mechanisms and gene-expression patterns would be identified that contribute toward elucidation of the virulence-enhancing effects catecholamines have on *E. coli* O157:H7.

Materials and methods

Microorganism and growth conditions

Eight separate replicates of the study were performed on 4 separate days. For each replicate, a single isolated colony was derived from glycerol stocks of ATCC 43895 cryo-stock by plating on tryptic soy agar. Isolated colonies were inoculated and grown overnight in tryptic soy broth. From the same overnight culture, 200 μL ($\sim 2.0 \times 10^7$ CFU mL^{-1}) were used to inoculate 10 mL aliquots of preincubated (serum-SAPI media was preincubated, for 24 h to stabilize temperature and gas equilibrium) serum-SAPI minimal medium (Lyte *et al.*, 1996a, 1997) containing either 50 μM norepinephrine in phosphate-buffered saline for treatment samples or the same volume of vehicle for control samples. EDL933 (ATCC 43895) isolates were cultured at 37 °C, 0.05% CO_2, 95% humidity on a rocking platform at 0.5 cycle s^{-1}. All cultures were maintained in Falcon six-well tissue culture plates (Becton Dickinson, NJ) to ensure gas exchange within the cultures were optimized over the 5-h incubation. After 5 h incubation, cells were harvested directly into bacteria protect RNA solution (Qiagen Inc.) and processed for RNA extraction and subsequent microarray analyses.

Microarray design

Using the transcriptome of *E. coli* O157:H7 EDL933, an oligonucleotide microarray, made up of 43–45 mers, was designed. A total of 610 genes (including 10 negative control probes derived from pig mRNA sequences) were selected. Designed using a custom script, specifications of oligos were based on various design characteristics such as temperature of melting, 3′ location, specificity, lack of repeat nucleotides, etc. (Charbonnier *et al.*, 2005). Oligonucleotides were synthesized and normalized in concentration by Integrated DNA Technologies Inc. (Coralville, IA). Oligos were resuspended in Epoxide Spotting Solution and printed onto Epoxide Coated Slides (Corning Inc., Corning, NY) using a GeneMachine's Omnigrid Accent microarray printer (Genomic Solutions, Ann Arbor, MI). Each array consisted of duplicate elements for each gene and each slide contained a duplicate array with spot sizes of 170 μm, separated at a distance of 300 μm. MIAME information for this array is contained within the NCBI GEO under accession GPL3935.

Microarray protocol

All procedures were performed according to respective manufacturer protocols. Total RNA was extracted using the RNeasy Bacteria Mini Kit (Qiagen Inc.) and trace amounts of DNA were removed using the RNase-Free DNase Set (Qiagen Inc.). RNA was quantified using spectrophotometry (Nanodrop) and quality confirmed by electrophoresis. For each sample, 10 μg of RNA were labeled with either CyDye3-dCTP or CyDye5-dCTP (Amersham Biosciences) using the LabelStar kit (Qiagen Inc.) and Random nonamers (Sigma-Aldrich Inc., St Louis, MO). Labeled cDNA were hybridized to the microarray using the Pronto Universal Hybridization Kit and the Short Oligo Hybridization Solution (Corning Inc.). A total of 16 arrays, each with duplicate elements for each gene, including a dye swap for each replicate (eight biological replicates), was analyzed to obtain genes that were consistently regulated while limiting a false discover rate (FDR) below 5% (Benjamini & Hochberg, 1995).

Microarray analysis

Microarray images were captured using a Genepix 4000B (Molecular Devices Corporation, Union City, CA) laser scanner and images processed using GENEPIX 6.0 software (Molecular Devices Corporation). Microarray data analyses were performed using ACUITY 4.0 software (Molecular Devices Corporation). Slides were normalized using standard methods and analyzed based on log ratio (635/532) values. Genes were included in the final data set that exhibit at least a |1.5|-fold comparative regulation.

Quantitative PCR

Quantitative PCR results for six induced and repressed genes were used as a validation of the microarray results. Measurements of relative transcript amounts were performed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) with the QuantiTect SYBR Green RT-PCR kit (Qiagen Inc.) according to the manufacturer's instructions. Cycling conditions were based on the standard settings recommended for the 7500 Sequence Detection System (PE Applied Biosystems). In all cases the same RNA samples used for cDNA synthesis and microarrays were subsequently used for quantitative PCR validation. Specific primer pairs were designed using APPLIED BIOSYSTEMS PRIMER SELECT Software and standard settings (PE Applied Biosystems). The genes selected for final analyses using qRT-PCR included *stx*2a (forward 5′-TTCGCGCCGTGAATGAA; reverse 5′-CAGGCCTGTCGCCAGTTATC), *stx*1B (forward 5′-TGCATCGCTTTCATTTTTTTCA; reverse 5′-CCACC

TTTCCAGTTACACAATCAG), *eae* (forward 5′-CGGAT AACGCCGATACCATT; reverse 5′-GCCTGAGCTACCC CATTCTTT), *esp*B (forward 5′-TCGCAGAACGCAGGAT GATA; reverse 5′-GCGGACAGCGGACGTTATAT), *esp*A (forward 5′-GCCAAAATTGCTGATGTTCAGA; reverse 5′-CGTCTTGAGGAAGTTTGGCTTT), and *psp*A. Their amplification efficiency was estimated to be between 89% and 95%. In addition, the 16S RNA gene (forward 5′-CCAGGGCTACACACGTGCTA; reverse 5′-TCTCGC GAGGTCGCTTCT) transcript was analyzed for both control and treatment samples, in order to normalize the qRT-PCR results of selected genes. The reactions were performed on an ABI Prism 7500 Sequence Detection system (PE Applied Biosystems). The difference (fold) in the initial concentration of each transcript (normalized to 16S rRNA gene) with respect to the control was calculated according to the comparative C_t method using the built-in functions of the 7500 SYSTEM SEQUENCE DETECTION Software version 1.3 (Applied Biosystems). The results of the qRT-PCR are provided in Table 2 and correlated with microarray expression (correlation coefficient 0.99, $P < 0.0001$).

Statistics

Correlation analyses were performed using multivariate analyses functions of JMP 6.0 (SAS Institute, Cary, NC). Power analysis was performed using k sample means function of JMP 6.0 (SAS institute). Built-in functions of 7500 SYSTEM SEQUENCE DETECTION Software version 1.3 (Applied Biosystems) were used to average replication C_t for quantitative PCR. On the basis of power analyses, a total of 16 arrays, each with duplicate elements for each gene, including a dye swap for each replicate (eight biological replicates), was analyzed to obtain genes that were consistently regulated while limiting FDR below 5% (Benjamini & Hochberg, 1995). Statistical calculations related to microarray expression were performed within ACUITY 4.0 (Molecular Devices Corporation) and confirmed by a separate analysis using GENESPRING 6.0 (Agilent Technologies).

Results and discussion

EDL933 (ATCC 43895) isolates were cultured at 37 °C in the presence and absence of norepinephrine in a serum-SAPI-based media under 0.05% CO_2, 95% humidity environment, with inoculums of $\sim 2.2 \times 10^6$ CFU mL^{-1} (Lyte *et al.*, 1996a, 1997). A lower inoculum would be more biologically relevant, but this variable had to be balanced with the need to isolate sufficient RNA concentrations to use for microarray hybridizations. Similarly, an additional variable related to growth stage could have been alleviated by allowing the control culture to grow longer but this would have added a time variable that is not entirely rational in terms of evaluating pathogenesis. Thus, a 5 h time point was chosen to evaluate RNA expression in response to norepinephrine. This time frame is also relevant in relation to transit time and time to clinical infection within the GI tract (Sarmiento *et al.*, 1988; Cray & Moon, 1995; Shere *et al.*, 2002; Chen *et al.*, 2003; La Ragione *et al.*, 2005). The difference in $OD_{600\,nm}$ after 5-h of incubation was only 0.012 ($OD_{600\,nm}$ norepinephrine–$OD_{600\,nm}$ control). As noted in previous studies on growth effects of norepinephrine in serum-SAPI, the earliest norepinephrine that can increase growth is between 4 and 6 h (Lyte *et al.*, 1996b; Chen *et al.*, 2006), and (M. Lyte, pers. commun.) thus this study, which evaluates the norepinephrine-influenced genetic profile at 5 h, is appropriate for furthering the intended goals.

It was hoped that using a 610-gene microarray, described and validated previously (Dowd & Ishizaki, 2006), would corroborate previous findings (Lyte *et al.*, 1996a, b, 1997; Collington *et al.*, 1998; Freestone *et al.*, 2000) and reveal novel regulatory mechanisms that contribute to norepinephrine-associated pathogenesis. The results (Table 1) showed significant differential regulation (FDR < 5%) of 101 genes, and corroborated well with previous findings related to the predominant virulence factors including *stx1*, *stx2*, and *eae* (Lyte *et al.*, 1996a; Green *et al.*, 2004; Vlisidou *et al.*, 2004; Chen *et al.*, 2006). The most highly induced genes in the norepinephrine-exposed isolates were the locus of enterocyte effacement (LEE)-secreted protein (*esp*AB) transcripts. Iron binding and transport proteins were also highly induced, although novel findings showed that transcripts related to iron scavenging such as e*nt*DC and *fep*AC were comparatively repressed. The most highly repressed genes were a group of phage shock proteins *psp*A-E. There was a variety of regulatory genes including *yih*L, *rse*AB, *pho*B, nusBG, *gln*G, *ybb*U, *psp*C, and *fur* that were comparatively repressed while induced transcription regulation genes included *rpo*N, rcsA, *gcv*A, *icc*, *qse*B, *msb*B, and *rst*A (Table 1).

Virulence factors

The induction of *stx1*, *stx2*, and LEE-associated transcripts, in the presence of norepinephrine, has been discussed in detail (Lyte & Bailey, 1997; Lyte *et al.*, 1996a, 1997; Vlisidou *et al.*, 2004). The concurrence of the results of this study with previous observations also lends validation to the current data set. Thus, conditions that result in elevated norepinephrine and a subsequent spillover of this catecholamine into the iron-deplete environment of the GI (acute stress responses) could result in enhanced pathogenesis of *E. coli* O157:H7 (Chen *et al.*, 2003; Green *et al.*, 2004) by induction of the major virulence factors associated with its pathogeneses.

The LEE region of the EHEC O157:H7 strain EDL933 (Perna *et al.*, 2001) indicates the presence of 41 ORFs, most

Table 1. Genes significantly regulated during growth in the presence of norepinephrine

Name	Definition	Fold regulation	Benjamini–Hochberg (FDR)
pspB	Phage shock protein, putative inner membrane protein	−14.8	0.00
pspA	Phage shock protein, putative inner membrane protein	−14.2	0.00
pspD	Phage shock protein	−14.1	0.00
pspC	Phage shock protein: activates phage shock-protein expression	−12.5	0.01
cyoE	Protoheme IX farnesyltransferase (heme O biosynthesis)	−4.8	0.00
glnG	Response regulator for gln (sensor glnL) (nitrogen regulator I, NRI)	−4.3	0.01
yihM	orf, hypothetical protein	−3.8	0.00
yihL	Putative transcriptional regulator GntR	−3.7	0.00
ompF	Outer membrane protein 1a (Ia;b;F)	−3.6	0.00
pspE	Phage shock protein	−3.1	0.00
rseB	Regulates activity of sigma-E factor	−2.6	0.00
fur	Negative regulator	−2.3	0.04
secD	Protein secretion; membrane protein, part of the channel	−2.3	0.00
fepA	Outer membrane receptor for ferric enterobactin (enterochelin) and colicins B and D	−2.2	0.03
hmpA	Dihydropteridine reductase, ferrisiderophore reductase activity	−2.2	0.00
csgG	Curli production assembly/transport component, second curli operon	−2.1	0.00
htpG	Chaperone Hsp90, heat shock protein C 62.5	−2.1	0.01
fliE	Flagellar biosynthesis; basal-body component, possibly at (MS-ring)-rod junction	−2.1	0.00
rpoE	RNA polymerase, sigma-E factor; heat shock and oxidative stress	−2.0	0.00
ybbU	Putative regulator	−2.0	0.00
entC	Isochorismate hydroxymutase 2, enterochelin biosynthesis	−2.0	0.00
rseA	Sigma-E factor, negative regulatory protein	−2.0	0.02
phoB	Positive response regulator for pho regulon, sensor is PhoR (or CreC)	−1.9	0.00
fepC	ATP-binding component of ferric enterobactin transport	−1.9	0.03
nusB	Transcription termination; L factor	−1.8	0.01
nupC	Permease of transport system for three nucleosides	−1.8	0.04
glnS	Glutamine tRNA synthetase	−1.8	0.00
csgF	Curli production assembly/transport component, second curli operon	−1.7	0.00
dnaJ	Chaperone with DnaK; heat shock protein	−1.7	0.00
Omexp	Putative outer membrane export protein	−1.7	0.03
lysP	Lysine-specific permease	−1.7	0.00
kdtA	3-deoxy-D-manno-octulosonic-acid transferase (KDO transferase)	−1.7	0.00
secF	Protein secretion, membrane protein	−1.7	0.02
entD	Enterobactin synthetase component D	−1.6	0.05
nusG	Component in transcription antitermination	−1.6	0.01
invasin4	Putative invasin	−1.6	0.01
fsr	Fosmidomycin resistance protein	−1.6	0.02
ompG	Outer membrane protein	−1.6	0.02
aroP	Aromatic amino acid transport protein	−1.6	0.01
secE	Preprotein translocase	−1.6	0.01
sfmF	Putative fimbrial protein	−1.6	0.02
ptrB	Protease II	−1.6	0.02
qseA	Quorum sensing *Escherichia coli* regulator A	−1.5	0.05
spoT	(p)ppGpp synthetase II; also guanosine-3′, 5′-bis pyrophosphate 3′-pyrophosphohydrolase	−1.5	0.00
marB	Multiple antibiotic resistance protein	1.5	0.01
QseC	Quorum sensing *Escherichia coli* regulator C	1.5	0.05
icc	Regulator of lacZ	1.5	0.01
hemD	Uroporphyrinogen III synthase	1.5	0.00
def	Peptide deformylase pdf	1.5	0.02
rcsC	Sensor for ctr capsule biosynthesis, probable histidine kinase acting on RcsB	1.6	0.01
msbB	Suppressor of htrB, heat shock protein	1.6	0.01
umuD	SOS mutagenesis; error-prone repair; processed to UmuD′; forms complex with UmuC	1.6	0.05
recB	DNA helicase, ATP-dependent dsDNA/ssDNA exonuclease V subunit, ssDNA endonuclease	1.6	0.00
fimE	Recombinase involved in phase variation; regulator for fimA	1.6	0.05
inhcelldiv	Putative inhibitor of cell division encoded by cryptic prophage CP-933M	1.6	0.03
slyA	Transcriptional regulator for cryptic hemolysin	1.6	0.00
slp	Outer membrane protein induced after carbon starvation	1.6	0.00

Table 1. Continued.

Name	Definition	Fold regulation	Benjamini–Hochberg (FDR)
phoQ	Sensor protein PhoQ	1.6	0.01
rcsA	Positive regulator for ctr capsule biosynthesis, positive transcription factor	1.6	0.05
ddg	Putative heat shock protein	1.7	0.03
rfaH	Transcriptional activator affecting biosynthesis of lipopolysaccharide core, F pilin, and haemolysin	1.7	0.03
gadA	Glutamate decarboxylase isozyme	1.7	0.04
degQ	Serine endoprotease	1.7	0.00
fimA	Putative minor fimbrial subunit	1.7	0.02
cutF	Copper homeostasis protein (lipoprotein)	1.8	0.03
gcvA	Positive regulator of gcv operon	1.8	0.00
wecF	TDP-Fuc4NAc:lipidII transferase; synthesis of enterobacterial common antigen (ECA)	1.8	0.01
plsC	Putative phospholipid biosynthesis acyltransferase	1.8	0.00
osmB	Osmotically inducible lipoprotein	1.8	0.03
wecC	UDP-*N*-acetyl-D-mannosaminuronic acid dehydrogenase; synthesis of ECA	1.9	0.02
fecC	Putative iron compound permease protein of ABC transporter family	1.9	0.05
relA	(p)ppGpp synthetase I (GTP pyrophosphokinase)	1.9	0.05
rstA	Response transcriptional regulatory protein (RstB sensor)	1.9	0.00
hemC	Porphobilinogen deaminase = hydroxymethylbilane synthase	2.0	0.00
holin2	Putative holin protein of prophage CP-933O	2.0	0.00
fepC	Putative ATP-binding protein of ABC transporter family	2.1	0.01
rpoN	RNA polymerase, sigma(54 or 60) factor; nitrogen and fermentation regulation	2.1	0.00
fpr	Ferredoxin-NADP reductase	2.1	0.00
malG	Part of maltose permease, inner membrane	2.1	0.05
Omptin	Outer membrane protein 3b (a), protease VII	2.1	0.02
qseB	Quorum sensing *Escherichia coli* regulator B	2.3	0.02
wza	Putative polysaccharide export protein	2.5	0.01
gadB	Glutamate decarboxylase isozyme	2.5	0.03
feoB	Ferrous iron transport protein B	2.6	0.00
chuU	Putative permease of iron compound ABC transport system	2.7	0.00
stx2A	Shiga-like toxin II A subunit encoded by bacteriophage BP-933W	2.7	0.00
fhuD	Putative iron compound-binding protein of ABC transporter family	2.7	0.00
endolys3	Putative endolysin of prophage CP-933O	2.7	0.00
fecD	Putative iron compound permease protein of ABC transporter family	2.7	0.01
fimb5	Putative minor fimbrial subunit	2.8	0.01
wzx	O antigen flippase Wzx	2.8	0.04
ibpB	Heat shock protein	3.0	0.00
cspG	Homolog of *Salmonella* cold shock protein	3.4	0.00
cspH	Cold shock-like protein	3.6	0.02
endolys1	Putative endolysin of cryptic prophage CP-933M	3.8	0.00
etpC	EtpC	3.9	0.01
ibpA	Heat shock protein	5.3	0.00
eae	Intimin adherence protein	5.4	0.02
tagA	ToxR-regulated lipoprotein	5.6	0.03
stx1B	Shiga-like toxin 1 subunit B encoded within prophage CP-933V	7.1	0.03
espB	Secreted protein EspB	13.8	0.01
espA	Secreted protein EspA	14.5	0.02

Details for those genes that showed differential regulation > 1.5 while minimizing FDR below 5%. Fold regulation is provided in terms of norepinephrine-grown cultures and based on log ratio (632/532). Thus, negative values are comparatively repressed while positive values are comparatively induced in norepinephrine-grown isolates.

of which are organized into several operons (Elliott *et al.*, 1998). The *ler* gene, which is encoded in the *LEE1* operon, has been determined to be the essential activator of the *LEE*-related operons (Elliott *et al.*, 2000) and the *hha* gene, which is a negative regulator of hemolysin genes (Nieto *et al.*, 1991), has been associated with repression effects on *ler* transcription and subsequent LEE expression (Sharma & Zuerner, 2004). In the array data of this study, the *hha* gene showed no notable regulation. Although *hha* was not repressed, which is reported among many other mechanisms to lead to LEE induction, hemolysin transcription regulators were induced. This could provide a logical fit that better coordinates LEE expression with iron bioavailability. The transcripts for *sly*A (regulator of hemolysin expression) and

Table 2. Quantitative RT-PCR results

Gene	qRT-PCR fold regulation	Microarray fold regulation
eae	2.5	5.4
*esp*B	7.12	13.8
*psp*A	−4.7	−14
*stx*1b	4.9	7
*esp*A	7.2	14.4
*stx*2a	2.5	2.7

*rfa*H (transcriptional activator of hemolysin) were significantly induced. RfaH acts as an antiterminator for the expression of the same hemolysin operons repressed by *hha* (Leeds & Welch, 1996). This suggests inherent regulatory interaction with iron metabolism and iron bioavailability and provides a plausible explanation for LEE induction under the current growth conditions. There are many regulatory factors that have implications in the control of LEE as reviewed by Walters & Sperandio (2006); hence there is little elucidation to be gained, other than indicating how the current results do, or do not, concur with previous reports. It is evident that *qse*A is repressed, which is a noted positive regulator of LEE (Daubin *et al*., 2003) yet *qse*BC were induced. Additionally, *gad*A is induced, which is a gene under positive regulation of *gad*X, which has also been reported to be a positive regulator of LEE expression (Shin *et al*., 2001) but was not regulated. Finally, the repression of *spo*T, but the induction of *rel*A and *rcs*C, indicates that there might be a stringent response activation of LEE (Tobe *et al*., 2005; Nakanishi *et al*., 2006). Thus, both concurrence and disagreement with known mechanisms of LEE induction exist. However, as each of these same systems are also intimately tied in with the iron regulon (Gaille *et al*., 2003; Kim *et al*., 2005; Vinella *et al*., 2005; Laaberki *et al*., 2006; Muller *et al*., 2006; Diggle *et al*., 2007) it would seem logical that iron bioavailability might be playing a primary governing role in LEE regulation, explaining its induction in the presence of norepinephrine in the current experiment as discussed below.

Iron acquisition and transport

Norepinephrine has been shown to promote the uptake of iron that is complexed with lactoferrin or transferrin (Freestone *et al*., 2000). Data revealed both induction and repression of many transcripts involved in iron transport and scavenging, respectively. There were five transcripts, induced during growth with norepinephrine, that participate in the ATP-binding cassette iron transport system *fhu*D, *fep*C, *fec*CD, and *chu*U. Additionally, the *feo*B gene, which encodes an outer membrane transporter of ferric iron, the *fpr* gene, which encodes a ferredoxin-NADP reductase, and *hem*CD transcripts, which are primary genes involved in heme biosynthesis, are induced. The induction of iron transport and utilization genes is contrasted by the comparative repression of enterobactin (iron scavenging)-associated genes including *cyo*E, *fep*AC, *hmp*A, and *ent*CD. It has been shown that *E. coli* O157 uses enterobactin, in a reaction facilitated by the presence of norepinephrine, to obtain iron from transferrin molecules (Freestone *et al*., 2003). Thus, the comparative repression of the enterobactin-related genes, in the presence of norepinephrine, may seem contradictory in the light of those findings (Freestone *et al*., 2003), yet it would be expected that norepinephrine levels were not actually regulating these genes directly. It is expected that the lack of iron bioavailability in the control culture induces expression of iron-scavenging genes. In other words, it is to be expected that norepinephrine increases bioavailability of iron, which under these conditions results in a comparative repression of those genes associated with iron scavenging.

In the norepinephrine-grown isolates *spo*T, which encodes guanosine-3′, 5′-bis pyrophosphate 3′-pyrophospho (ppGpp) hydrolase was repressed while *rel*A (ppGpp synthase) is induced. In *E. coli*, ppGpp acts as a positive and negative regulator of many transcripts. RelA is a ppGpp synthase and SpoT is both a hydralase and a synthase (Vinella *et al*., 2005). Although there are implications in LEE induction, the ppGpp molecule is best known in its role of promoting glutamate and lysine production in cells (Imaizumi *et al*., 2006); however, it also acts as a universal stress regulatory factor (Braeken *et al*., 2006). In control cultures, *spo*T may have been repressed and *rel*A induced in response to iron bioavailability, which would be yet another unique induction factor for this noted multitasking regulatory molecule (Traxler *et al*., 2006). Supporting this regulatory interaction, recent research (Vinella *et al*., 2005) has shown that ppGpp exerts a form of positive control on iron uptake. This fits well with the current model in which norepinephrine increases iron bioavailability in this model system. Also of note, a target for future research was the repression of *fur*, which has been a well studied and primary regulator of the very complex iron regulon. Recent findings show that the *fur* gene in *Helicobacter pylori* is vital to the adaptation, not only to low iron concentration but also to the relatively low pH encountered in the host upper GI environment (Gancz *et al*., 2006). This provides a notable link to the ongoing microbial endocrinology (Lyte, 1993) research related to gastric dopamine and its possible virulence-enhancing effects on enteric pathogens.

Acid resistance (AR) and SOS

AR in *E. coli* strains such as O157:H7 contribute to the low infectious dosages of these organisms. Survival of the passage through the stomach is facilitated by at least three different AR mechanisms that include an *rpo*S-dependent system and a glutamate decarboxylase system. Notable

among the genes induced in response to norepinephrine are *gad*AB, which are the primary genes involved in the glutamate decarboxylase AR system. These two genes are thought to impart AR by the consumption of protons during decarboxylation of arginine and glutamate (Castanie-Cornet *et al.*, 2007). In addition, *rcs*ABC are induced (*rcs*B = 1.2-fold; $P = 0.01$), which have also been shown to take part in the glutamate AR pathway (Castanie-Cornet *et al.*, 2007). This is the first indication that norepinephrine might enhance AR.

The induction of *csp*G, *ibp*AB, and *stx*2 and other genes associated with the bacterial stress response is of interest. In relation to the expression of *ibp*AB, the bacterial stress-response factor sigma(54) was induced. Sigma(54) is noted to be a positive regulator of *ibp*AB (Kuczynska-Wisnik *et al.*, 2001). However, another of the primary operons reported to be under its positive control (*psp*A-E) was repressed. These genes encode the phage shock protein (psp) system (Jovanovic *et al.*, 1996). The psp system is reported to respond to extracytoplasmic stress (Darwin, 2005) and the conditions that modulate the *psp* operon have been associated with alterations in proton motive force (PMF) including acidic conditions. This suggests that there is a dramatic difference in PMF in norepinephrine-exposed cultures potentially tied to repression of large bore porins such as *omp*F, *sec*DEF, *aro*P, and *nup*C.

In bacteria, the Sec proteins provide a route for proteins to cross the cytoplasmic membrane and for insertion of OMP such as *omp*F (Driessen *et al.*, 2001). It has even been suggested that SecD and SecF function together to stimulate OMP export (Darwin, 2005) and have also been specifically associated with alterations in PMF (Nouwen *et al.*, 2001) but were not noted to be responsible for PMF maintenance. The *omp*F-encoded protein has been noted to be involved in the transport of large molecules across the membrane and its repression in norepinephrine cultures would obviously decrease diffusion of protons (increasing AR) and, as noted, greatly influence PMF (Yamada *et al.*, 1989). Investigations into how norepinephrine influences PMF are definitively warranted.

Finally, the results show induction of *umu*D, *stx*2, *rec*B, and lambdoid phage-encoded transcripts induction, which may also indicate an SOS response initiated in response to norepinephrine exposure (Keller *et al.*, 2001; Bjedov *et al.*, 2003; Hare *et al.*, 2006). The *umu*D gene regulates *rec*A-dependent DNA repair SOS response (Hare *et al.*, 2006). The *rec*B gene is involved in SOS induction (Keller *et al.*, 2001) and because the *stx*2 genes are located on a lambdoid phage its expression is also indicative of an SOS response (Plunkett, III *et al.*, 1999). Thus, it is now being investigated whether norepinephrine might be an inducer of a novel positive adaptive state (Dowd *et al.*, 2007) in *E. coli* O157, which could have dramatic implications in pathogenesis (LeClerc & Cebula, 2000).

The experimental design is largely consistent with previous studies using these model systems and as such provides both corroborative and novel data. As with many microarray studies, these data provide several new directions expanding the scope of microbial endocrinology out of iron bioavailability. A panel of new observations is made here, especially related to PMF, SOS response, and AR. Yet, it must also be acknowledged that with any such study a population of bacteria rather than one single bacterium is normalized. In addition, the results obviously do not represent the complete influence of norepinephrine on gene expression in *E. coli* O157:H7, because they reflect a single analysis at a single time point in a single model system. Also of note is the potential effect of differential growth rate on gene expression. At 5 h postinoculation, the norepinephrine-treated cultures may start dividing more rapidly than the control cultures. Thus, it is important to clarify that the attempt is made here only to evaluate gene expression differences related to exposure of O157 to norepinephrine. Research must now be directed to determine whether there are gene expression changes in relation to the presence of norepinephrine that occur ahead of (are not related to) those changes specific to increased iron bioavailability and enhanced growth rate.

USDA disclaimer

The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the United States Department of Agriculture.

Acknowledgements

Thanks to Jason Williams and Dr Chunfa Wu for their expert technical assistance. Thanks to Mark Lyte for helpful discussions and critical review of the manuscript.

References

Aneman A, Eisenhofer G, Olbe L, Dalenback J, Nitescu P, Fandriks L & Friberg P (1996) Sympathetic discharge to mesenteric organs and the liver. Evidence for substantial mesenteric organ norepinephrine spillover. *J Clin Invest* **97**: 1640–1646.

Benjamini Y & Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Statist Soc B* **57**: 289–300.

Bjedov I, Tenaillon O, Gerard B, Souza V, Denamur E, Radman M, Taddei F & Matic I (2003) Stress-induced mutagenesis in bacteria. *Science* **300**: 1404–1409.

Bowdre JH, Krieg NR, Hoffman PS & Smibert RM (1976) Stimulatory effect of dihydroxyphenyl compounds on the

aerotolerance of *Spirillum volutans* and *Campylobacter fetus* subspecies *jejuni*. *Appl Environ Microbiol* **31**: 127–133.

Braeken K, Moris M, Daniels R, Vanderleyden J & Michiels J (2006) New horizons for (p)ppGpp in bacterial and plant physiology. *Trends Microbiol* **14**: 45–54.

Castanie-Cornet MP, Treffandier H, Francez-Charlot A, Gutierrez C & Cam K (2007) The glutamate-dependent acid resistance system in *Escherichia coli*: essential and dual role of the His-Asp phosphorelay RcsCDB/AF. *Microbiology* **153**: 238–246.

Charbonnier Y, Gettler B, Francois P, Bento M, Renzoni A, Vaudaux P, Schlegel W & Schrenzel J (2005) A generic approach for the design of whole-genome oligoarrays, validated for genomotyping, deletion mapping and gene expression analysis on *Staphylococcus aureus*. *BMC Genomics* **6**: 95.

Chen C, Brown DR, Xie Y, Green BT & Lyte M (2003) Catecholamines modulate *Escherichia coli* O157:H7 adherence to murine cecal mucosa. *Shock* **20**: 183–188.

Chen C, Lyte M, Stevens MP, Vulchanova L & Brown DR (2006) Mucosally-directed adrenergic nerves and sympathomimetic drugs enhance non-intimate adherence of *Escherichia coli* O157:H7 to porcine cecum and colon. *Eur J Pharmacol* **539**: 116–124.

Collington GK, Booth IW, Donnenberg MS, Kaper JB & Knutton S (1998) Enteropathogenic *Escherichia coli* virulence genes encoding secreted signalling proteins are essential for modulation of Caco-2 cell electrolyte transport. *Infect Immun* **66**: 6049–6053.

Costa M, Brookes SJ & Hennig GW (2000) Anatomy and physiology of the enteric nervous system. *Gut* **47**: (Suppl 4): iv15–iv19.

Cray WC Jr & Moon HW (1995) Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7. *Appl Environ Microbiol* **61**: 1586–1590.

Darwin AJ (2005) The phage-shock-protein response. *Mol Microbiol* **57**: 621–628.

Daubin V, Moran NA & Ochman H (2003) Phylogenetics and the cohesion of bacterial genomes. *Science* **301**: 829–832.

Diggle SP, Matthijs S, Wright VJ *et al.* (2007) The *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. *Chem Biol* **14**: 87–96.

Dowd SE & Ishizaki H (2006) Microarray based comparison of two *Escherichia coli* O157:H7 lineages. *BMC Microbiol* **6**: 30.

Dowd SE, Killinger-Mann K, Blanton J, San Francisco M & Brashears M (2007) Adaptive state: microarray evaluation of gene expression in *Salmonella enterica* Typhimurium upon exposure to sub-therapeutic levels of nalidixic acid. *Foodborne Pathog Dis* **4**: 40–50.

Driessen AJ, Manting EH & van der DC (2001) The structural basis of protein targeting and translocation in bacteria. *Nat Struct Biol* **8**: 492–498.

Eisenhofer G, Aneman A, Hooper D, Holmes C, Goldstein DS & Friberg P (1995) Production and metabolism of dopamine and norepinephrine in mesenteric organs and liver of swine. *Am J Physiol* **268**: G641–G649.

Elliott SJ, Wainwright LA, McDaniel TK, Jarvis KG, Deng YK, Lai LC, McNamara BP, Donnenberg MS & Kaper JB (1998) The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic *Escherichia coli* E2348/69. *Mol Microbiol* **28**: 1–4.

Elliott SJ, Sperandio V, Giron JA, Shin S, Mellies JL, Wainwright L, Hutcheson SW, McDaniel TK & Kaper JB (2000) The locus of enterocyte effacement (LEE)-encoded regulator controls expression of both LEE- and non-LEE-encoded virulence factors in enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect Immun* **68**: 6115–6126.

Freestone PP, Haigh RD, Williams PH & Lyte M (1999) Stimulation of bacterial growth by heat-stable, norepinephrine-induced autoinducers. *FEMS Microbiol Lett* **172**: 53–60.

Freestone PP, Lyte M, Neal CP, Maggs AF, Haigh RD & Williams PH (2000) The mammalian neuroendocrine hormone norepinephrine supplies iron for bacterial growth in the presence of transferrin or lactoferrin. *J Bacteriol* **182**: 6091–6098.

Freestone PP, Haigh RD, Williams PH & Lyte M (2003) Involvement of enterobactin in norepinephrine-mediated iron supply from transferrin to enterohaemorrhagic *Escherichia coli*. *FEMS Microbiol Lett* **222**: 39–43.

Gaille C, Reimmann C & Haas D (2003) Isochorismate synthase (PchA), the first and rate-limiting enzyme in salicylate biosynthesis of *Pseudomonas aeruginosa*. *J Biol Chem* **278**: 16893–16898.

Gancz H, Censini S & Merrell DS (2006) Iron and pH homeostasis intersect at the level of Fur regulation in the gastric pathogen *Helicobacter pylori*. *Infect Immun* **74**: 602–614.

Green BT, Lyte M, Kulkarni-Narla A & Brown DR (2003) Neuromodulation of enteropathogen internalization in Peyer's patches from porcine jejunum. *J Neuroimmunol* **141**: 74–82.

Green BT, Lyte M, Chen C, Xie Y, Casey MA, Kulkarni-Narla A, Vulchanova L & Brown DR (2004) Adrenergic modulation of *Escherichia coli* O157:H7 adherence to the colonic mucosa. *Am J Physiol Gastrointest Liver Physiol* **287**: G1238–G1246.

Hare JM, Perkins SN & Gregg-Jolly LA (2006) A constitutively expressed, truncated umuDC operon regulates the recA-dependent DNA damage induction of a gene in *Acinetobacter baylyi* strain ADP1. *Appl Environ Microbiol* **72**: 4036–4043.

Imaizumi A, Kojima H & Matsui K (2006) The effect of intracellular ppGpp levels on glutamate and lysine overproduction in *Escherichia coli*. *J Biotechnol* **125**: 328–337.

Jones RK & Shapiro AP (1963) Increased susceptibility to pyelonephritis during acute hypertension by angiotensin II and norepinephrine. *J Clin Invest* **42**: 179–186.

Jovanovic G, Weiner L & Model P (1996) Identification, nucleotide sequence, and characterization of PspF, the transcriptional activator of the *Escherichia coli* stress-induced psp operon. *J Bacteriol* **178**: 1936–1945.

Keller KL, Overbeck-Carrick TL & Beck DJ (2001) Survival and induction of SOS in *Escherichia coli* treated with cisplatin, UV-irradiation, or mitomycin C are dependent on the function of the RecBC and RecFOR pathways of homologous recombination. *Mutat Res* **486**: 21–29.

Kim EJ, Wang W, Deckwer WD & Zeng AP (2005) Expression of the quorum-sensing regulatory protein LasR is strongly affected by iron and oxygen concentrations in cultures of *Pseudomonas aeruginosa* irrespective of cell density. *Microbiology* **151**: 1127–1138.

Kinney KS, Austin CE, Morton DS & Sonnenfeld G (2000) Norepinephrine as a growth stimulating factor in bacteria – mechanistic studies. *Life Sci* **67**: 3075–3085.

Kuczynska-Wisnik D, Laskowska E & Taylor A (2001) Transcription of the ibpB heat-shock gene is under control of sigma(32)- and sigma(54)-promoters, a third regulon of heat-shock response. *Biochem Biophys Res Commun* **284**: 57–64.

Laaberki MH, Janabi N, Oswald E & Repoila F (2006) Concert of regulators to switch on LEE expression in enterohemorrhagic *Escherichia coli* O157:H7: Interplay between Ler, GrlA, HNS and RpoS. *Int J Med Microbiol* **296**: 197–210.

La Ragione RM, Ahmed NM, Best A, Clifford D, Weyer U, Cooley WA, Johnson L, Pearson GR & Woodward MJ (2005) Colonization of 8-week-old conventionally reared goats by *Escherichia coli* O157:H7 after oral inoculation. *J Med Microbiol* **54**: 485–492.

LeClerc JE & Cebula TA (2000) *Pseudomonas* survival strategies in cystic fibrosis. *Science* **289**: 391–392.

Leeds JA & Welch RA (1996) RfaH enhances elongation of *Escherichia coli* hlyCABD mRNA. *J Bacteriol* **178**: 1850–1857.

Lyte M (1993) The role of microbial endocrinology in infectious disease. *J Endocrinol* **137**: 343–345.

Lyte M (2004a) Microbial endocrinology and infectious disease in the 21st century. *Trends Microbiol* **12**: 14–20.

Lyte M (2004b) The biogenic amine tyramine modulates the adherence of *Escherichia coli* O157:H7 to intestinal mucosa. *J Food Prot* **67**: 878–883.

Lyte M & Bailey MT (1997) Neuroendocrine-bacterial interactions in a neurotoxin-induced model of trauma. *J Surg Res* **70**: 195–201.

Lyte M & Ernst S (1992) Catecholamine induced growth of gram negative bacteria. *Life Sci* **50**: 203–212.

Lyte M & Nguyen KT (1997) Alteration of *Escherichia coli* O157:H7 growth and molecular fingerprint by the neuroendocrine hormone noradrenaline. *Microbios* **89**: 197–213.

Lyte M, Arulanandam BP & Frank CD (1996a) Production of Shiga-like toxins by *Escherichia coli* O157:H7 can be influenced by the neuroendocrine hormone norepinephrine. *J Lab Clin Med* **128**: 392–398.

Lyte M, Frank CD & Green BT (1996b) Production of an autoinducer of growth by norepinephrine cultured *Escherichia coli* O157:H7. *FEMS Microbiol Lett* **139**: 155–159.

Lyte M, Arulanandam B, Nguyen K, Frank C, Erickson A & Francis D (1997) Norepinephrine induced growth and expression of virulence associated factors in enterotoxigenic and enterohemorrhagic strains of *Escherichia coli*. *Adv Exp Med Biol* **412**: 331–339.

Monjan AA & Collector MI (1977) Stress-induced modulation of the immune response. *Science* **196**: 307–308.

Muller CM, Dobrindt U, Nagy G, Emody L, Uhlin BE & Hacker J (2006) Role of histone-like proteins H-NS and StpA in expression of virulence determinants of uropathogenic *Escherichia coli*. *J Bacteriol* **188**: 5428–5438.

Nakanishi N, Abe H, Ogura Y, Hayashi T, Tashiro K, Kuhara S, Sugimoto N & Tobe T (2006) ppGpp with DksA controls gene expression in the locus of enterocyte effacement (LEE) pathogenicity island of enterohaemorrhagic *Escherichia coli* through activation of two virulence regulatory genes. *Mol Microbiol* **61**: 194–205.

Nieto JM, Carmona M, Bolland S, Jubete Y, dela CF & Juarez A (1991) The hha gene modulates haemolysin expression in *Escherichia coli*. *Mol Microbiol* **5**: 1285–1293.

Nouwen N, van der LM & Driessen AJ (2001) SecDFyajC is not required for the maintenance of the proton motive force. *FEBS Lett* **508**: 103–106.

Perna NT, Plunkett G III, Burland V *et al.* (2001) Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature* **409**: 529–533.

Plunkett G III, Rose DJ, Durfee TJ & Blattner FR (1999) Sequence of Shiga toxin 2 phage 933W from *Escherichia coli* O157:H7: Shiga toxin as a phage late-gene product. *J Bacteriol* **181**: 1767–1778.

Sarmiento JI, Casey TA & Moon HW (1988) Postweaning diarrhea in swine: experimental model of enterotoxigenic *Escherichia coli* infection. *Am J Vet Res* **49**: 1154–1159.

Sharma VK & Zuerner RL (2004) Role of hha and ler in transcriptional regulation of the esp operon of enterohemorrhagic *Escherichia coli* O157:H7. *J Bacteriol* **186**: 7290–7301.

Shere JA, Kaspar CW, Bartlett KJ, Linden SE, Norell B, Francey S & Schaefer DM (2002) Shedding of *Escherichia coli* O157:H7 in dairy cattle housed in a confined environment following waterborne inoculation. *Appl Environ Microbiol* **68**: 1947–1954.

Shin S, Castanie-Cornet MP, Foster JW, Crawford JA, Brinkley C & Kaper JB (2001) An activator of glutamate decarboxylase genes regulates the expression of enteropathogenic *Escherichia coli* virulence genes through control of the plasmid-encoded regulator, Per. *Mol Microbiol* **41**: 1133–1150.

Tobe T, Ando H, Ishikawa H, Abe H, Tashiro K, Hayashi T, Kuhara S & Sugimoto N (2005) Dual regulatory pathways integrating the RcsC-RcsD-RcsB signalling system control enterohaemorrhagic *Escherichia coli* pathogenicity. *Mol Microbiol* **58**: 320–333.

Traxler MF, Chang DE & Conway T (2006) Guanosine 3′, 5′-bispyrophosphate coordinates global gene expression during glucose-lactose diauxie in *Escherichia coli*. *Proc Natl Acad Sci USA* **103**: 2374–2379.

Vinella D, Albrecht C, Cashel M & D'Ari R (2005) Iron limitation induces SpoT-dependent accumulation of ppGpp in *Escherichia coli*. *Mol Microbiol* **56**: 958–970.

Vlisidou I, Lyte M, van Diemen PM, Hawes P, Monaghan P, Wallis TS & Stevens MP (2004) The neuroendocrine stress hormone norepinephrine augments *Escherichia coli* O157:H7-induced enteritis and adherence in a bovine ligated ileal loop model of infection. *Infect Immun* **72**: 5446–5451.

Walters M & Sperandio V (2006) Autoinducer 3 and epinephrine signaling in the kinetics of locus of enterocyte effacement gene expression in enterohemorrhagic *Escherichia coli*. *Infect Immun* **74**: 5445–5455.

Yamada H, Matsuyama S, Tokuda H & Mizushima S (1989) A high concentration of SecA allows proton motive force-independent translocation of a model secretory protein into *Escherichia coli* membrane vesicles. *J Biol Chem* **264**: 18577–18581.